Journal of Medical Virology 58:387–393 (1999)

Nucleotide Sequence of Thymidine Kinase Gene of Sequential Acyclovir-Resistant Herpes Simplex Virus Type 1 Isolates Recovered From a Child With Wiskott–Aldrich Syndrome: Evidence for Reactivation of Acyclovir-Resistant Herpes Simplex Virus

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Recurrent acyclovir (ACV)-resistant (ACV-r) herpes simplex virus type 1 (HSV-1) infections occurred in a patient with Wiskott–Aldrich syndrome, an X-linked recessive immunodeficiency syndrome composed of three clinical characteristics of immunodeficiency, thrombocytopenia, and an eczematous dermatitis. The patient had severe and recurrent ACV-r herpes simplex and was treated with vidarabine in a satisfactory manner from 1993 to 1997. During the 4-year observation period, two ACV-sensitive (ACV-s) HSV-1 isolates and five ACV-r HSV-1 isolates were recovered. The nucleotide sequence of the thymidine kinase (TK) gene from these sequential ACV-r isolates was compared with the ACV-s isolates. A single nucleotide deletion of cytosine (C) from homopolymer stretch of four C residues between nucleotide 1061 and 1064 of the open reading frame was found in all ACV-r isolates. No other differences were observed in the TK nucleotide sequence between ACV-s and ACV-r isolates. The TK nucleotide sequences of the two ACV-s isolates were identical to each other and those of the five ACV-r isolates were identical to one another. These results suggest that the ACV-r HSV-1 might have derived from the ACV-s strain in the patient body and that TK-associated ACV-r HSV-1 can reactivate from latency. ***J. Med. Virol. 58:387–393, 1999.***

KEY WORDS: herpes simplex virus type 1; acyclovir; resistant; thymidine kinase; reactivation

INTRODUCTION

Recurrent herpes simplex virus (HSV) infections are frequent in patients with immunodeficiency. Although they are self-limiting in immunocompetent hosts, such infections may result in the severe form or in frequent relapse of herpes simplex.

9-(2-Hydroxyethoxy)-methylguanine (acyclovir, ACV) is widely used for the treatment of HSV or varicella zoster virus (VZV) infections [Elion et al., 1977; Schaeffer et al., 1978; Chou et al., 1981; Straus et al., 1982; Wade et al., 1982; Dorsky and Crumpacker, 1987]. As a result of the wide use of ACV in immunocompromised patients, the incidence of ACV-resistant (ACV-r) herpes simplex virus types 1, 2 (HSV-1 and HSV-2, respectively) and VZV has been increasing [Sibrack et al., 1982; Pahwa et al., 1988; Chatis et al., 1989; Erlich et al., 1989; Englund et al., 1990; Jacobson et al., 1990; Ljungman et al., 1990; Mondiano et al., 1995]. Most of ACV-r HSV or VZV have been revealed to be either viral thymidine kinase (TK)-deficient (TK^-) [De Clercq, 1985; Sasadeusz et al., 1997] or substrate altered TK (TK^A) [Darby et al., 1981; Ellis et al., 1981; Larder et al., 1983]. Clinical isolates of ACV-r HSV with a mutation in the DNA polymerase gene have been reported as a different mechanism for their resistance to ACV from TK-associated ACV resistance [Collins et al., 1989].

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Accepted 18 December 1998

It is reported that TK^- and ACV-r HSV mutants can establish latency in mouse trigeminal ganglia but can not reactivate [Coen et al., 1989b; Efstathiou et al., 1989; Tenser et al., 1989]. The expression of the viral TK gene plays no major role in the establishment of the latent state, but it has a role during reactivation from latency in neural ganglia. Recently, reactivation of TK^- and ACV-r HSV-2 in a patient with acquired immunodeficiency syndrome (AIDS) was reported [Palú et al., 1992].

We witnessed the recurrent ACV-r HSV-1 disease such as severe herpes simplex, in a boy with Wiskott–Aldrich syndrome (WAS), a congenital immunodeficiency syndrome composed of the triad, immunodeficiency, thrombocytopenia, and an eczematous dermatitis. We reported that the ACV-r HSV-1 recovered from this patient with WAS lost the ability to phosphorylate ACV at all by the alteration of TK activity [Saijo et al., 1998].

In this article, the TK gene of the sequential HSV-1 isolates from the patient from March 1993 to March 1997 was studied at a molecular level. We report here evidence of reactivation from latency of TK-associated ACV-r HSV-1 in a patient with immunodeficiency.

METHODS

Clinical History

The clinical course on the HSV-1 infections in a boy with WAS has been reported previously [Saijo et al., 1998]. Briefly, the patient had primary HSV-1 infection at the age of 3. Since that time, he had suffered from recurrent and severe herpes simplex, mostly treated with intravenous or oral administration of ACV. At age 8 in March 1993, a severe HSV-1 mucocutaneous infection occurred around his left eye, which was treated with continuous administration of ACV (2 mg/kg per hour): the lesion improved in 6 weeks. His recurrent HSV-1 skin infections were treated with ACV at the dose of 80 mg/kg per day (divided into four doses, orally) for prophylaxis.

In May 1994, severe herpes simplex appeared on his face, arm, genitalia, back, and foot. ACV was administered continuously (2 mg/kg per hour), but the skin lesions did not respond. HSV-1 was isolated from the skin lesions and was tested for sensitivity to ACV in human embryonic lung fibroblast cells using the plaque reduction assay. The 50% inhibitory concentration (IC_{50}) of ACV to the isolate was 36.0 μg/ml, demonstrating that the isolate was resistant to ACV. Subsequently, the patient was treated with vidarabine, and the ACV-r herpes simplex showed improvement.

After the improvement of ACV-r herpes simplex in March 1994, the prophylactic use of oral ACV was stopped, resulting in the recurrent relapses of herpes simplex. The relapse of herpes simplex was treated with intravenous or oral administration of ACV, when the virus isolates were demonstrated to be susceptible to ACV.

The patient had five episodes of ACV-r herpes simplex outbreaks since March 1994. Each time it was treated with continuous, high dose ACV-therapy or with combined therapy with continuous administrations of ACV (2 mg/kg per hour) and vidarabine (15 mg/kg per day), resulting in improvement. Assessment of each ACV-r herpes simplex was based not only on the clinical symptoms but also on the virus isolation results.

Virus Isolation and Cultivation

Clinical specimens were obtained from mucocutaneous lesions with cotton swabbing and were inoculated onto Vero (African green monkey kidney) or human embryonic lung fibroblast (HEL) cells. The cells were cultured until typical cytopathic effect was evident. Viral identification was performed on infected cell cultures using direct immunofluorescent method using Microtrak Herpes Direct Test™ (SYVA, San Francisco, CA). HSV-1 isolates were subsequently grown in Vero cells and used at passage levels 2 and 3.

Viruses

One laboratory HSV-1 strain (KOS) and nine clinical HSV-1 isolates were used for the TK gene nucleotide sequence analysis to evaluate the diversity of HSV-1 TK nucleotide sequence among HSV-1 isolates. The nine clinical isolates were recovered from nine patients with gingivostomatitis.

Compounds

ACV (Sigma Chemical Company, St. Louis, MO), 9-β-D-arabinofuranosyladenine (vidarabine; Mochida Pharmaceutical Co. Ltd., Tokyo, Japan), phosphonoformic acid (foscarnet; Sigma) were used.

Sensitivity to Antiviral Agents

The sensitivity of isolates to antiviral agents was assayed by the plaque reduction method in Vero cells as described previously [Saijo et al., 1992]. Isolates were regarded as being sensitive if the 50 % inhibitory concentration (IC_{50}) of ACV, vidarabine and foscarnet for isolates were under 2, 20, and 100 μg/ml, respectively. In the case that the IC_{50} of the respective antiviral compounds for the isolates was over 2, 20, and 100 μg/ml, they were regarded as being resistant.

Phosphorylation of ACV in ACV-s-, ACV-r, or Mock-Infected Vero Cells by Thin Layer Chromatography (TLC)

To confirm that the ACV resistance of HSV-1 isolates recovered from this patient was associated with lack of ACV-phosphorylation activity of viral TK, the phosphorylation pattern of ACV in virus-infected Vero cells was assayed by TLC. The method of TLC was the same as in previous reports [Votruba et al., 1987; Sakuma et al., 1991; Saijo et al., 1992] . Briefly, Vero cells were infected with the ACV-s isolate, ACV-r isolate, or mock at a multiplicity of infection of 2 and were incubated for 1 hr. After the incubation, each virus solution was removed. Then each virus-infected Vero cells were cultivated in Eagle's minimal essential medium containing

2% fetal bovine serum (MEM-FBS2) for 12 hr. Subsequently, the cells were cultivated in MEM-FBS2 containing ^{3}H-ACV (Sigma) at a concentration of 37 KBq/ml and were incubated for 4 hr. The acid-soluble fraction of each virus-infected Vero cells was extracted and assayed for ACV phosphorylation pattern by TLC.

Anti-Serum Against HSV-1 TK for Western Blotting Analysis

Anti-HSV-1 TK rabbit serum was produced as described below. DNA of HSV-1 TAS-infected Vero cells [Saijo et al., 1998] were purified by the Hirt's extraction method. PCR was performed with 2 ng of template and 50 pmol of each primer TKf(Bam) 5′-TAGAGGATCCTATGGCTTCGTACCCCTG-3′ and TKr(Hind) 5′-CCAAGCTTCAGTTAGCCTCCCCCATCTC, which had Bam HI and Hind III restriction site, respectively, using Expand™ Hi-Fidelity PCR System (Boehringer Mannheim Biochemica, Tokyo, Japan). The amplification condition was as follows: denaturing at 100°C for 5 min; 5 cycles of denaturing at 94°C for 40 sec, annealing at 40°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C at 2 min; 15 cycles of denaturing at 94°C for 40 sec, annealing at 55°C for 1 min, extension at 72°C for 2 min; additional extension at 72°C for 5 min. The amplified TK DNA was digested with Bam HI and Hind III and was then subcloned into Bam HI and Hind III restriction sites within the multiple cloning site of pQE31 plasmid (QIAGEN, GmbH, Hilden, Germany). *Escherichia coli* (BL21 strain) was transformed with pQE31 plasmid that codes for 6× His-tagged HSV-1 TK. The 6×His-tagged HSV-1 TK was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) and was expressed in *E. coli.* The expressed 6× His-tagged HSV-1 TK was purified on Ni-NTA agarose (QIAGEN, GmbH, Hilden, Germany). Rabbits were immunized three times with a mixture of the purified HSV-1 TK and a Freund's adjuvant. The serum, which contained high titer of anti-HSV-1 TK antibody, was collected.

Western Blotting Analysis of TK Polypeptide of ACV-s and ACV-r Isolates

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of ACV-s or ACV-r HSV-1 isolates recovered from this patient were carried out. Each virus-infected Vero cells were washed with phosphate-buffered saline (PBS) solution (pH 7.4), were suspended in PBS solution, and were homogenized by sonication. The samples were then adjusted to 1× SDS sample buffer (0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) and were boiled for 10 min. Equal quantities of each sample were loaded onto an SDS-10% polyacrylamide gel with 4% stacking gel. Protein bonds were then electroblotted onto nitrocellulose membrane. The blots were then inoculated with PBS containing 0.05% Tween 20 and 5% skim milk for 1 hr at room temperature to block nonspecific sites. The blots were then inoculated with the appropriate primary antibody (anti-HSV-1 TK rabbit serum at 1:1000) for 1 hr at room temperature. After washing of the membrane, the membrane was inoculated with secondary antibody, a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1000) (Kirkegaard & Perry Laboratories Inc., Baltimore, MD); antigen levels were detected by a POD substrate (Wako BioProducts, Inc., Tokyo, Japan).

DNA Sequencing

DNA sequencing of the TK gene was performed by cycle sequencing of PCR-generated products amplified from purified viral DNA from Vero cells infected with each isolate. DNA purification was performed with Hirt's extraction method from each isolate-infected Vero cells after incubation until there was a greater than 80% cytopathic effect from inoculation of each isolate to confluent Vero cells (multiplicity of infection, 0.1).

PCR was performed with 2 ng of template and 50 pmol of each primer external to the TK open reading frame (ORF) (S6f 5′-ACAGCGTGCCGCAGATCTTG-3′ and S1r 5′-TATCGACAGAGTGCCAGCCC-3′) using Expand™ Hi-Fidelity PCR System to amplify the entire TK gene and its boundary sequences. Amplification condition included denaturing at 100°C for 5 min, 30 cycles of denaturing at 94°C for 40 sec, annealing at 60°C for 1 min, and extension at 72°C for 40 sec, and then an extension step at 72°C for 10 min. After electrophoretic separation and visualization with ethidium bromide in 1% agarose, the PCR product was purified with a QIAquick PCR Purification Kit™ (QIAGEN GmbH, Hilden, Germany). Cycle sequencing using dye-labeled terminators was performed with four overlapping sense internal primers, TKpf 5′-CCCGCACCTCTTTGGCAAGCG-3′, TKf(0) 5′-ATGGCTTCGTACCCCTGCCATCAACACG-3′, TKf(392) 5′-CTTATGCCGTGACCGACGCC-3′, and TKf(782) 5′-AGCTTTCGGGGACGGCCGTG-3′ to sequence the entire TK ORF and its boundary lesions. Reactions were carried out with dRhodamine Dye Terminator Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) according to the manufacture's instructions. The reaction conditions included denaturing at 96°C for 3 min, 25 cycles of denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec, and extension at 60°C for 4 min. The extension products in the reaction mixture were then purified with the spin column purification method using a Centri-Sep Spin Column (PE Applied Biosystems). Products were then sequenced with ABI PRISM™ 310 Genetic Analyser (PE Applied Biosystems) and were then confirmed by automated sequencing.

RESULTS

Virus Isolates

During the 4-year-observation period, seven isolates of HSV-1 were recovered from the patient with WAS and recurrent herpes simplex. The date of isolation and site of lesions are listed in Table I. The isolates were

TABLE I. Characteristics of the Sequential HSV-1 Isolates

Isolates	Isolation dates	Isolation sites	Sensitivity to antiviral agents		
			ACV	Vidarabine	Foscarnet
HSV-1 TA1	March 1993	Left periorbital	S (0.17*)	S (4.4)	S (17)
HSV-1 TA2	May 1994	Perianal	R (36)	S (2.9)	S (18)
HSV-1 TA3	November 1994	Left periorbital	S	S	S
HSV-1 TA4	March 1995	Left periorbital	R	S	S
HSV-1 TA5	March 1996	Left periorbital	R	N.T.	N.T.
HSV-1 TA6	December 1996	Perianal	R	N.T.	N.T.
HSV-1 TA7	March 1997	Perioral	R	N.T.	N.T.

ACV, acyclovir; S, sensitive; R, resistant; N.T., not tested.
*The values in parentheses are IC_{50} (μg/ml) of each antiviral agent for the TA1 and TA2 isolates.

recovered from his facial and anogenital lesions as documented previously [Saijo et al., 1998].

Sensitivity Test

HSV-1 TA1 and TA3 were sensitive to ACV, vidarabine, and foscarnet. On the other hand, TA2 and TA4-7 strains were resistant to ACV, but sensitive to vidarabine and foscarnet. The sensitivity pattern of TA1 and TA3 isolates indicated that they express the TK polypeptide with intact activity. However, the sensitivity pattern of the other isolates (TA2 and TA4-7) suggests that they do not express the TK polypeptide with ACV-phosphorylation activity.

ACV Phosphorylation Pattern in the ACV-s and ACV-r HSV-1 Isolates

To confirm that the resistance of the ACV-r HSV-1 isolates recovered from this patient was due to lack of ACV-phosphorylation activity by viral TK, the phosphorylation pattern of ACV was evaluated by TLC. ACV-triphosphate (ACV-TP) was detected in ACV-s HSV-1 TA1. However, ACV-TP was not detected in ACV-r HSV-1 TA2-infected Vero cells (Fig. 1). There was no difference in the phosphorylation pattern of ACV between ACV-r HSV-1 TA2- and mock-infected Vero cells. These results revealed that HSV-1 TA2 isolate acquired resistance to ACV by the loss of ACV phosphorylation activity. These results suggested that some mutations of TK gene occurred in ACV-r HSV-1 TA2 isolate.

Sequence of TK Gene of Isolates

In an attempt to identify the genetic basis for the resistance to ACV in sequential isolates, the TK gene of all seven isolates was sequenced. The complete nucleotide and amino acid sequences of HSV-1 TA1 and TA2 TK genes are shown in Figure 2. Single deletion of cytosine (C) from homopolymer stretch of 4 C residues between nucleotide 1061 and 1064 of the ORF was confirmed in all ACV-r isolates (TA2 and TA4-7) in comparison with ACV-s isolates (TA1 and TA3) (Fig. 2). No other differences were observed in the TK and its boundary nucleotide sequence between ACV-r and ACV-s isolates. Whereas the TK polypeptide of ACV-s HSV-1 TA1 and TA3 is composed of 376 amino acid, the TK polypeptide of the ACV-r isolates is expected to be composed of 407 amino acids according to its nucleotide sequence. We confirmed that the TK polypeptide of ACV-r HSV-1 TA2 was bigger, as was expected, than that of ACV-s HSV-1 TA1 by Western blotting method (Fig. 3). The amino acid sequence of ACV-r isolates changed from the 355 amino acid to C′-terminal in comparison with that of ACV-s isolates. The TK nucleotide sequences of ACV-s isolates "TA1 and TA3" were identical to each other. The sequences of ACV-r isolates "TA2 and TA4-7" were also identical to one another. These results provide an evidence that in human TK-associated ACV-r, HSV-1 can reactivate from latency.

Diversity of HSV-1 TK Nucleotide Sequence in HSV-1 Clinical Isolates in Japan

Besides HSV-1 TA1-7, nine HSV-1 clinical isolates recovered from nine Japanese children and one laboratory strain KOS were determined of TK nucleotide sequence to evaluate the diversity of TK nucleotide sequence among HSV-1 clinical isolates. The difference in TK nucleotide sequence of the total 10 HSV-1 (1 laboratory strain and 9 clinical isolates) was summarized in comparison with that of HSV-1 TA1 strain on the Table II. One clinical isolate, 96-586, showed the completely identical TK nucleotide sequence with that of HSV-1 TA1. However, the other 9 HSV-1 had the different TK nucleotide sequence from that of HSV-1 TA1. The numbers of different nucleotides from that of HSV-1 TA1 were between 0 and 7 in HSV-1 TK nucleotide sequence of the 10 HSV-1 strains (KOS strain and 9 clinical isolates) (Table II).

DISCUSSION

The present paper deals with the genetic characterization of seven sequential isolates recovered from the same patient during a long period from March 1993 to March 1997. Palú et al. [1992] presented the first report in which 6 HSV-2 isolates, sequentially recovered from ulcerative anogenital lesions of an AIDS patient during a prolonged treatment with ACV, was studied at the molecular level. Their ACV-r HSV-2 was shown to be virtually deficient in TK activity due to the production of truncated TK polypeptide. In their report, they stated that TK^- and ACV-r HSV-2 sequentially recovered from the same patient had the same molecular characteristics, indicating the possibility of reactivation from latency of TK^- ACV-r HSV-2 in immunocompromised patients. They speculated that the reac-

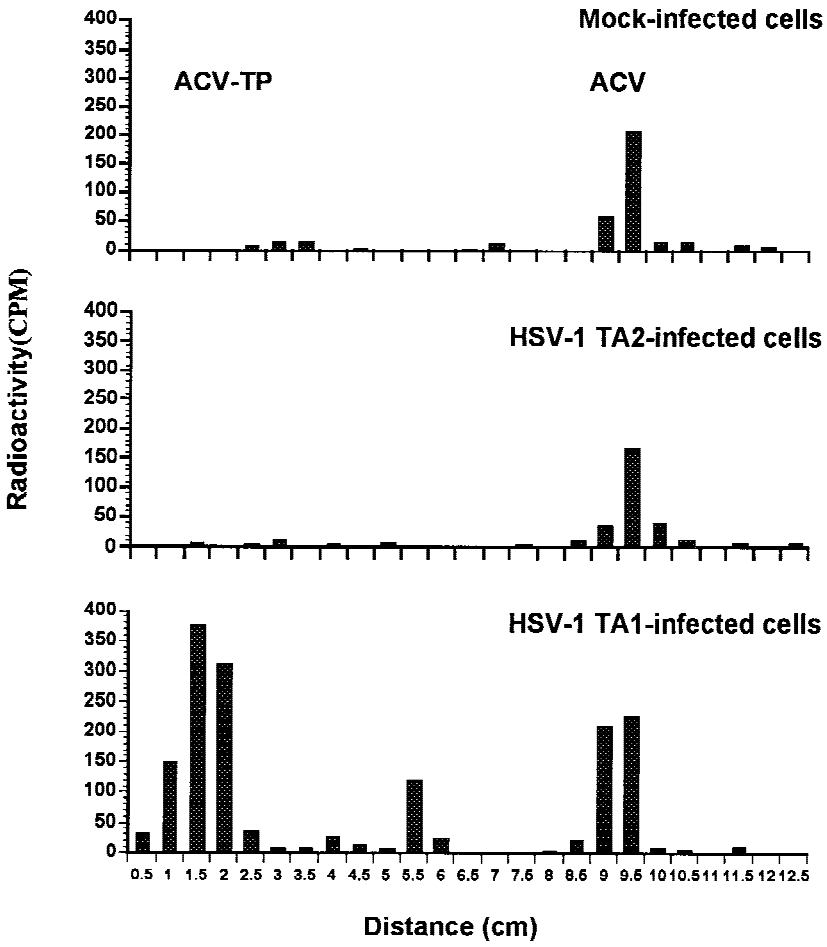

Fig. 1. Phosphorylation of acyclovir (ACV) in herpes simplex virus type 1 (HSV-1) TA1- **(lower)**, HSV-1 TA2- **(middle)**, and mock- **(upper)** infected Vero cells by thin layer chromatography.

tivation of TK^- HSV-2 might be through a helper TK-positive (TK^+) virus.

We showed by TLC that HSV-1 TA2 acquired resistance to ACV by the loss of ACV phosphorylation activity by viral TK, indicating that the resistance to ACV was associated with some mutations in viral TK polypeptide. The nucleotide sequence of the ACV-r isolates was determined and was compared with that of the ACV-s isolates (Fig. 2). Furthermore, the size of the TK polypeptide of HSV-1 TA2 was compared with that of HSV-1 TA1 by Western blotting method (Fig. 3).

In the present study, two major conclusions were reached. First, ACV-r isolates were identical in nucleotide sequence of TK gene, indicating that all ACV-r isolates originated from the same strain. Second, one point mutation, a single deletion of cytosine (C) from homopolymer stretch of 4 C residues between nucleotide 1061 and 1064 of the ORF, was detected in TA2 and TA4-7 isolates in comparison with that of both TA1 and TA3 isolates. This evidence suggests that the ACV-r HSV-1 isolate was derived from the ACV-s isolate during a long-term administration of ACV for prophylaxis from March 1993 to April 1994. The latter conclusion is supported by the result of diversity of HSV-1 TK nucleotide sequence among clinical isolates recovered from Japanese children (Table II). Based on these conclusions, TK-associated ACV-r HSV-1 has the ability to reactivate from latency and to cause recurrent ACV-r herpes simplex virus infections in immunocompromised hosts.

A frameshift mutation, resulting from a single deletion of C from the homopolymer stretch of 4 C residues between nucleotide 1061 and 1064 of the ORF in TK gene of ACV-s isolates, was found in the ACV-r isolates (TA2 and TA4-7). As a result, a TK polypeptide with a longer amino acid sequence (407 aa), with an altered amino acid sequence of carboxy-terminal portion (355–407 aa), was generated (Figs. 2, 3). The mechanism of resistance of the HSV-1 with this TK polypeptide is under investigation. So far, ACV-r herpes viruses, which are associated with deficient TK activity, are reported to have mutated amino acid substitution at the ATP-binding site in the TK polypeptide [Liu and Summers, 1988] or to have frameshift mutation resulting in a production of a truncated TK polypeptide [Sawyer et al., 1988; Coen et al., 1989a; Palú et al., 1992]. TK mutants characterized by an altered substrate specificity or by a diminished enzymatic activity have

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ATG GCT TCG TAC CCC TGC CAT CAA CAC GCG TCT GCG TTC GAC CAG GCT GCG CGT TCT CGC   60   TA1-7
 M   A   S   Y   P   C   H   Q   H   A   S   A   F   D   Q   A   A   R   S   R   20
GGC CAT AGC AAC CGA CGT ACG GCG TTG CGC CCT CGC CGG CAG CAA GAA GCC ACG GAA GTC  120   TA1-7
 G   H   S   N   R   R   T   A   L   R   P   R   R   Q   Q   E   A   T   E   V   40
CGC CTG GAG CAG AAA ATG CCC ACG CTA CTG CGG GTT TAT ATA GAC GGT CCT CAC GGG ATG  180   TA1-7
 R   L   E   Q   K   M   P   T   L   L   R   V   Y   I   D   G   P   H   G   M   60
GGG AAA ACC ACC ACC ACG CAA CTG CTG GTG GCC CTG GGT TCG CGC GAC GAT ATC GTC TAC  240   TA1-7
 G   K   T   T   T   T   Q   L   L   V   A   L   G   S   R   D   D   I   V   Y   80
GTA CCC GAG CCG ATG ACT TAC TGG CAG GTG CTG GGG GCT TCC GAG ACA ATC GCG AAC ATC  300   TA1-7
 V   P   E   P   M   T   Y   W   Q   V   L   G   A   S   E   T   I   A   N   I  100
TAC ACC ACA CAA CAC CGC CTC GAC CAG GGT GAG ATA TCG GCC GGG GAC GCG GCG GTG GTA  360   TA1-7
 Y   T   T   Q   H   R   L   D   Q   G   E   I   S   A   G   D   A   A   V   V  120
ATG ACA AGC GCC CAG ATA ACA ATG GGC ATG CCT TAT GCC GTG ACC GAC GCC GTT CTG GCT  420   TA1-7
 M   T   S   A   Q   I   T   M   G   M   P   Y   A   V   T   D   A   V   L   A  140
CCT CAT ATC GGG GGG GAG GCT GGG AGC TCA CAT GCC CCG CCC CCG GCC CTC ACC CTC ATC  480   TA1-7
 P   H   I   G   G   E   A   G   S   S   H   A   P   P   P   A   L   T   L   I  160
TTC GAC CGC CAT CCC ATC GCC GCC CTC CTG TGC TAC CCG GCC GCG CGA TAC CTT ATG GGC  540   TA1-7
 F   D   R   H   P   I   A   A   L   L   C   Y   P   A   A   R   Y   L   M   G  180
AGC ATG ACC CCC CAG GCC GTG CTG GCG TTC GTG GCC CTC ATC CCG CCG ACC TTG CCC GGC  600   TA1-7
 S   M   T   P   Q   A   V   L   A   F   V   A   L   I   P   P   T   L   P   G  200
ACA AAC ATC GTG TTG GGG GCC CTT CCG GAG GAC AGA CAC ATC GAC CGC CTG GCC AAA CGC  660   TA1-7
 T   N   I   V   L   G   A   L   P   E   D   R   H   I   D   R   L   A   K   R  220
CAG CGC CCC GGC GAG CGG CTT GAC CTG GCT ATG CTG GCC GCG ATT CGC CGC GTT TAC GGG  720   TA1-7
 Q   R   P   G   E   R   L   D   L   A   M   L   A   A   I   R   R   V   Y   G  240
CTG CTT GCC AAT ACG GTG CGG TAT CTG CAG GGC GGC GGG TCG TGG CGG GAG GAT TGG GGA  780   TA1-7
 L   L   A   N   T   V   R   Y   L   Q   G   G   G   S   W   R   E   D   W   G  260
CAG CTT TCG GGG ACG GCC GTG CCG CCC CAG GGT GCC GAG CCC CAG AGC AAC GCG GGC CCA  840   TA1-7
 Q   L   S   G   T   A   V   P   P   Q   G   A   E   P   Q   S   N   A   G   P  280
CGA CCC CAT ATC GGG GAC ACG TTA TTT ACC CTG TTT CGG GCC CCC GAG TTG CTG GCC CCC  900   TA1-7
 R   P   H   I   G   D   T   L   F   T   L   F   R   A   P   E   L   L   A   P  300
AAC GGC GAC CTG TAT AAC GTG TTT GCC TGG GCC TTG GAC GTC TTG GCC AAA CGC CTC CGT  960   TA1-7
 N   G   D   L   Y   N   V   F   A   W   A   L   D   V   L   A   K   R   L   R  320
CCC ATG CAC GTC TTT ATC CTG GAT TAC GAC CAA TCG CCC GCC GGC TGC CGG GAC GCC CTG 1020   TA1-7
 P   M   H   V   F   I   L   D   Y   D   Q   S   P   A   G   C   R   D   A   L  340

CTG CAA CTT ACC TCC GGG ATG GTC CAG ACC CAC GTC ACC ACC CCA GGC TCC ATA CCG ACG 1080   TA1, 3
CTG CAA CTT ACC TCC GGG ATG GTC CAG ACC CAC GTC ACC ACC CAG GCT CCA TAC CGA CGA        TA2,4-7
 L   Q   L   T   S   G   M   V   Q   T   H   V   T   T   P   G   S   I   P   T  360   TA1, 3
 L   Q   L   T   S   G   M   V   Q   T   H   V   T   T   Q   A   P   Y   R   R        TA2,4-7

ATC TGC GAC CTG GCG CGC ACG TTT GCC CGG GAG ATG GGG GAG GCT AAC TGA AAC ACG GAA 1140   TA1, 3
TCT GCG ACC TGG CGC GCA CGT TTG CCC GGG AGA TGG GGG AGG CTA ACT GAA ACA CGG AAG        TA2,4-7
 I   C   D   L   A   R   T   F   A   R   E   M   G   E   A   N Stop                  TA1, 3
 S   A   T   W   R   A   R   L   P   G   R   W   G   R   L   T   E   T   R   K  380   TA2,4-7

GGA GAC AAT ACC GGA AGG AAC CCG CGC TAT GAC GGC AAT AAA AAG ACA GAA TAA AAC GCA 1200   TA1, 3
GAG ACA ATA CCG GAA GGA ACC CGC GCT ATG ACG GCA ATA AAA AGA CAG AAT AAA ACG CAC        TA2,4-7
 E   T   I   P   E   G   T   R   A   M   T   A   I   K   R   Q   N   K   T   H  400   TA2,4-7

CGG GTG TTG GGT CGT TTG TTC ATA AAC GCG GGG TTC GGT CCC AGG GCT GGC ACT C 3'         TA1, 3
GGG TGT TGG GTC GTT TGT TCA TAA ACG CGG GGT TCG GTC CCA GGG CTG GCA CTC  3'           TA2,4-7
 G   C   W   V   V   C   S Stop                                                      TA2,4-7
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Fig. 2. The TK nucleotide and amino acid sequences of herpes simplex virus type 1 (HSV-1) TA1-7.

been shown to be caused by single nucleotide changes, mainly at the level of the nucleotide/nucleoside binding site [Chatis and Crumpacker, 1991; Nugier et al., 1991]. In this regard, this type of alteration in the TK polypeptide is different from "substitution" or "truncation." In addition to TK mutations, certain alterations in DNA polymerase region have been reported to result in ACV resistance [Collins et al., 1989]. The DNA polymerase activity of our seven isolates, which were recovered from this patient, was believed to be intact because of the sensitivity pattern to ACV, vidarabine, and foscarnet.

Substitution of amino acids at ATP- or nucleotide/nucleoside-binding sites or a truncated TK polypeptide

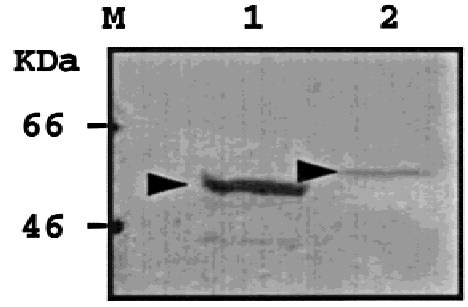

Fig. 3. Western blotting analysis of the thymidine kinase (TK) polypeptide of herpes simplex virus type 1 (HSV-1) TA1 **(lane 1)** and TA2 **(lane 2)**. M, marker.

TABLE II. Difference of HSV-1 KOS Strain and 9 HSV-1 Clinical Isolates in TK Nucleotide Sequence From That of HSV-1 TA1

HSV-1	Difference(s) in nucleotide sequence from that of HSV-1 TA1	No. of differences
KOS	C694T*, C1176A	2
96-586	None	0
I5-48	C348T	1
Il-6	C348T	1
I5-15	C802A, G1144A	2
96-435	C228A, C477G	2
I3-19	A580C, G1148A	2
I4-32	C578G, G631T, A1144G	3
I4-2	T16G, C22T, A24G, T125C, T171C, A266G, T849C	7
96-36	T16G, C22T, A24G, T125C, T171C, A266G, T849C	7

HSV-1, herpes simplex virus type 1; TK, thymidine kinase.
*"C694T" indicates that nucleotide "C" of HSV-1 TA1 was substituted to "T" at the position 694 in the TK open reading frame of KOS strain.

can result in ACV resistance. The present study on TK of the ACV-s and ACV-r HSV-1 isolates at the molecular level indicates that C′-terminal portion of TK polypeptide plays an important role in acquisition of resistance to ACV. However, the detailed mechanism of ACV resistance of this type of alteration in TK polypeptide other than "substitution" and "truncation" is not known.

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